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(54) **Pentaerythritol tetranitrate reductase enzyme**

(57) An enzyme capable of catalyzing the removal of nitrite from pentaerythritol tetranitrate (PETN) is provided. The enzyme is produced by culturing a novel strain of the Enterobacter cloacae bacterium isolated from nature. There is also provided a method of detecting the presence of PETN in a sample. These operate in the presence of NADPH and the NADP produced in the catalyzed reaction with PETN is detected, either by bioluminescence or amperometrically. There is further provided a method for the bioremedial treatment of an environment contaminated with PETN which involves inoculating the environment with a sample of the bacterium to produce the PETN reductase enzyme. The enzyme can also be used in a preparative method for isomers of pentaerythritol di- and tri-nitrates.

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Explosives Detection and Biodegradation

This invention relates to explosives detection and biodegradation and in particular to a novel enzyme isolated from a microorganism, to the microorganism which produces the enzyme, to a method of using the enzyme to catalyze the aerobic biodegredation of pentaerythritol tetranitrate (hereinafter referred to by the commonly used abbreviation PETN) and to a method and apparatus for the detection of PETN using the enzyme.

PETN has a diverse range of applications including as an explosive in blasting caps and detonators and in pharmaceutical compositions as the active ingredient of long-acting, slow onset coronary vasodilators for the prevention of angina attacks. The manufacture, handling and disposal of PETN can all lead to the contamination of the environment with PETN. There are concerns regarding the environmental fate of nitrate esters due to their relative recalcitrance and there therefore exists a need for a means of removing this contaminant from the environment without producing other undesirable pollutants. There is also an urgent requirement for a better method of detecting PETN as the currently proposed analytical systems rely mostly on use of bulky and sophisticated pieces of equipment such as gas chromatograms or mass spectrometers and/or require specially trained laboratory technicians for their application.

It is an aim of this invention to provide an enzyme which is capable of catalyzing the biodegradation of PETN and which may be employed in a bioremediation system for the environmental decontamination of the PETN pollutant.

According to a first aspect of the present invention therefore there is provided a PETN reductase enzyme which has the characteristics that, in the presence of nicotinamide adenine dinucleotide phosphate (NADPH hereinafter), it:-

1) catalyses the removal of nitrite from PETN; and

2) has reductase activity specifically at the nitrate ester linkage of PETN.

The action of the novel reductase enzyme of the present invention is to catalyze the reduction of PETN into pentaerythritol tri- and di-nitrates by attack at the nitrate ester linkage of PETN. The ability to specifically attack the nitrate ester linkage of PETN is a distinctive feature of this PETN reductase enzyme that is not possessed by commercially available reductases.

The ability of the novel PETN reductase enzyme to catalyse the removal of nitrite from PETN in the presence of NADPH allows the enzyme to be used in the detection of PETN. According to a second aspect of the invention therefore, there is provided a method of detecting the presence of PETN in a sample which comprises subjecting the sample to the PETN reductase enzyme of this invention in the presence of NADPH and under conditions to permit reaction of any PETN present in the sample and detecting the occurrence of such reaction. Conveniently, detection of the reaction would be by means of a bioluminescence test using either bacterial or firefly luciferase, as is well known in the art. Alternatively, the occurrence of reaction may be detected by an amperometric method as further discussed below.

In a third aspect, therefore, the present invention provides a biosensor for the detection of PETN in a sample which comprises means for contacting the sample with a PETN reductase enzyme in the presence of NADPH and means for detecting the occurrence of a reaction, catalyzed by the enzyme, of PETN when PETN is present in the sample. The means for detecting the occurrence of a reaction may conveniently comprise a bioluminescent transducer or an amperometric transducer. Such sensors can be used as the basis for highly convenient portable detectors for checking luggage, clothing etc. for traces of PETN.

The methods of making biosensors that rely on amperometric or bioluminescent changes in the test reaction are well known in the art. For example, UK Patent Application Publication No. 2,231,332A (NRDC) describes such methods and their use in biosensors, the contents of which in relation thereof is hereby incorporated by reference. Any of such

methods may be of use in detecting the occurrence of the PETN degrading reaction and hence in the detection of PETN.

Typically, in the case of a bioluminescent biosensor, luciferase is used. This enzyme is responsible for the light-emitting reaction of luminous bacteria and catalyzes the reaction of molecular oxygen with reduced flavin and aliphatic aldehyde to form long-lived intermediates whose slow breakdown provides energy to give light emission with reasonably high quantum yield. In coupling the PETN reductase to such a bioluminescent system, the $NADP^+$ generated through the activity of the PETN reductase is detected by the oxidation of an alcohol such as octanol or hexanol, to its corresponding aldehyde in the presence of an alcohol dehydrogenase. The aldehyde is then detected by reaction with reduced flavin ($FMNH_2$) in the presence of oxygen together with luciferase to catalyse the reaction. The intensity of the emitted light provides a measure of the $NADP^+$ converted to NADPH which in turn provides a measure of the PETN concentration present.

It is anticipated that using such a scheme, sensitivity to PETN will be in the range 0.1 nmol - 0.1μmol.

In an amperometric biosensor, the $NADP^+$ generated by reaction of the PETN reductase in the presence of NADPH may be re-reduced enzymatically with L-glutamate dehydrogenase (eg. from bovine liver), glutamate-pyruvate transaminase (eg. from pig heart) and pyruvate oxidase (eg. from Pediococcus sp.). The product is hydrogen peroxide and the concentration of this substance may be directly measured , as, for example, on a platinum electrode at 0.7v. or via a mediatorless peroxidase electrode.

The PETN reductase enzyme of this invention is characterised by the essential features described above but may be further identified by additional characteristics such as its pH optimum, catalytic activity, thermal stability or molecular weight. Details of such further characteristics are given in Example 3 below but it must be stressed that these characteristics are variable to a degree depending upon the conditions under which the microorganism producing the enzyme is grown and upon the degree of purification of the crude product. Variations of this kind will be well understood by those skilled in the art.

The PETN reductase enzyme of the present invention is obtained from a bacterial strain isolated from nature. The bacterial strain is a strain of *Pseudomonas alcaligenes* herein designated PB2. This novel bacterial isolate forms a further aspect of the present invention. A sample of the novel isolate has been deposited under the terms of the Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the purpose of patent procedures at the UK National Collection of Industrial and Marine Bacteria, 23 St Machar Drive, Aberdeen AB2 1RY, Scotland on the 14th April 1995 under deposit number NCIMB 40718.

According to a yet further aspect of the present invention, the ability of the PETN reductase enzyme to degrade PETN as described above provides a method for the bioremedial treatment of a PETN-contaminated environment which method comprises the step of inoculating the environment with a sample of the bacterial isolate of *Pseudomonas alcaligenes* designated PB2 and allowing the isolate to consume the PETN present in the environment. The environment concerned may be, for example, a waste stream of material containing PETN originating from the destruction of an explosives charge containing PETN or a sample of PETN-contaminated earth or other material. In the former case bioremedial treatment may be conveniently carried out in a reactor vessel, whereas in the latter instance the isolate may be introduced directly into the environment by inoculating the contaminated soil with it. Other appropriate methods of effecting treatment will be readily apparent to those skilled in the art.

The bacterium identified above will only produce the enzymic activity to which the present invention refers when it is cultured on PETN as the nitrogen source. Further characteristics of the deposited bacterium PB2 are listed below.

Gram stain		-ve
Spores		-ve
Motility		+ve
Growth	30°C	+ve
	41°C	+ve

45°C	+ve
Catalase	+ve
Oxidase	+ve
Fermentative in glucose	-ve
NO_3 reduction	+ve
Indole production	-ve
Acid from glucose	-ve
Arginine dihydrolase	-ve
Urease	-ve
Aesculin hydrolysis	-ve
Gelatin hydrolysis	-ve
β-galactosidase	-ve
Glucose assimilation	-ve
Arabinose assimilation	-ve
Mannose assimilation	-ve
N-acetylglucosamine assimilation	-ve
Maltose assimilation	-ve
Gluconate assimilation	+ve
Caprate assimilation	+ve
Adipate assimilation	+ve
Malate assimilation	+ve
Citrate assimilation	-ve
Phenylacetate assimilation	-ve
Cytochrome oxidase	+ve
Alkalisation of allantoin	+ve
Alkalisation of tartrate	+ve
Alkalisation of acetamide	-ve
NO_3 to NO_2	+ve
NO_3 to N_2	-ve
Utilisation of benzylamine	+ve
Utilisation of trehalose	+ve

Colony morphology:	Circular, regular, entire, smooth, raised, buff, semi-translucent. Yellow colour develops with age.

PETN reductase can be produced by culturing *P. alcaligenes* on PETN as a nitrogen source. When grown on NH_4NO_3 the activity can be induced by the addition of PETN. However, this is not the case when grown on NH_4Cl. Cultivation is preferably anoxic at any usual temperature, eg within 20 to 40°C range, preferably 25 to 30°C. To obtain PETN reductase the cells can be disrupted in any conventional way. Preferably, a cell free extract is made. The enzyme is then recovered from the extract.

Instead of the precise starting organism deposited, a mutant thereof, eg derived by gamma-ray irradiation or the use of a chemical mutant, induction by culture on another medium etc. or a transconjugant thereof with another bacterium or an artificially produced variant can be used. The ability of any such organism to give the enzymic activity can be readily determined by the skilled person.

The enzyme or some modification thereof can also be made by recombinant technology using methods well recognised in that art. These may entail producing the enzyme in another host organism.

In a further manner of use of the previously described activity of the PETN reductase of this invention, there is provided a method for producing isomers of pentaerythritol di- and tri-nitrates which may have useful pharmaceutical activity (biotransformation) by reacting PETN with the PETN reductase enzyme of the present invention, in the presence of NADPH.

The following Examples further illustrate the invention but are not to be regarded as limiting thereof.

EXAMPLE 1

Preparation of the enzyme activity from the bacterial strain Pseudomonas alcaligenes PB2

1.Materials and Methods

Pseudomanas alcaligenes PB2 was isolated using techniques standard in the art, from samples collected from a natural source by enrichment with PETN as the nitrogen source.

P. alcaligenes was grown in 1 litre of defined medium consisting of 10 mM potassium phosphate buffer, pH 7.3, containing 0.25 mM $MgS0_4$, 5 mM glucose, 5 mM succinate, 10 mM glycerol, 2mM NH_4NO_3, PETN (10 mM) and trace elements (as described by Pfennig and Lippert, Arch. Microbiology. 1966, 55, 726-739.) supplemented with $CaCl_2.2H_20$ (100mg/l).

Flasks were incubated at 180 r.p.m in a shaking incubator at 30°C. For bulk preparation of bacteria, 1 litre of seed culture was aseptically added to a 75 litre culture vessel, containing 50 litres of sterile medium. The bulk cultures were incubated at 30°C, stirred at 150 r.p.m. with sterile aeration to maintain dissolved oxygen at 10% saturation.

Cell free extracts were prepared from cells grown in the above manner. Cells were harvested from a 75 litre bulk culture, using continuous flow centrifugation (Sorval TZ-28 rotor, Sorval RC-5C centrifuge). Those obtained from a smaller volume culture were then pelleted by spinning at 10,000g for 15 min at 4°C in a Sorval RC-SC centrifuge fitted with a GS-3 rotor. These pelleted cells were resuspended in 2 ml of bis-Tris propane buffer (pH 7), per gram wet cell weight. Cells were disrupted by sonication in an MSE Soniprep (Fisons, Instruments, FSA Ltd.) using 6 x 12 μm bursts of 15 seconds, alternated with 30 seconds of cooling in melted ice. Cell debris and unbroken cells were removed by centrifugation at 20,000g for 20 min at 4°C in a Sorval RC-5C centrifuge using a SS-34 rotor, to give clarified cell free extract.

2 Chemicals

PETN was of the highest purity and other chemicals were of analytical grade, and unless stated otherwise, were obtained from BDH Ltd. (Poole, U.K), Sigma Chemical Company Ltd. (Poole, U.K) or Aldrich (Gillingham, U.K).

3 Assays

PETN reductase

PETN reductase activity was determined by monitoring the disappearance of PETN by HPLC in 50 mM bis-Tris propane buffer (pH 7), containing PETN (47 μM, final concentration), 40 μl enzyme and NADPH (0.2 mM, fmal concentration) in a final volume of 1 ml.

Alternatively, PETN degradation was also followed by monitoring the release of nitrite using Greiss reagent (Rosenblatt, Burrows, Mitchell and Parmer. 1991: "Organic Explosives and Related Compounds" in The Handbook of Environmental Chemistry 3 (G), edited by O.Hutzinger, Springer-Verlag). The assay was carried out as described above and terminated by the addition of ferricyanide (0.5 mM, final concentration) and phenazine methosulphate (0.2 mM, final concentration). Sulphanilic acid (0.6 mM, final concentration) was added and left to stand for 15 min. N-1-naphthylethylenediamine (0.4 mM, final concentration) was then added and after 5 min the colour which developed was measured spectrophotometrically at 540 nm. The unit enzyme activity is defined as the amount of enzyme necessary to release 1 μmol of nitrite per min at 30^{0}C.

The degradation of PETN could also be determined by monitoring the oxidation of NADPH at 340 nm.

Protein

Protein was routinely assayed by the Coomassie dye-binding method of Bradford (Anal. Biochem. (1976) 72, 248-254) using commercially available reagent and Bovine Serum Albumin standard (Pierce Ltd.- obtained through Life Science Labs Ltd., Luton).

An aliquate (20 µl) of sample containing 0.2-1 mg protein/ml was added to 1 ml of reagent and the reaction allowed to develop for 5 min at room temperature prior to reading the absorbance at 595 nm against a blank of buffer (20 µl) plus reagent (1 ml). Comparison to a standard curve of standard values (0-1 mg/ml) allowed calculation of the protein concentration in the sample.

Gel filtration standards

The following enzymes were used as molecular weight markers in gel filtration experiments: Bovine Serum albumen, Ovalbumen, Chymotrypsin and Ribonuclease A (molecular weights 67,000, 43,000, 25,000, 13,700 Daltons respectively).

EXAMPLE 2

Purification of PETN reductase

To the crude extract, obtained from 30g wet weight cells, enough ammonium sulphate was added to achieve 50% saturation. After stirring at 4°C for 5 min the resulting precipitate was removed by centrifugation at 20,000g for 20 min. Ammonium sulphate was added to the resulting supenatent to achieve 90% saturation. The resulting precipitate was collected by centrifugation at 4°C and redissolved in 4ml of 50 mM bis-Tris propane buffer (pH 7). The sample was desalted using a PD-10 column packed with Sephadex G-25M (Pharmacia) and concentrated to 8ml by ultrafiltration using an Amicon stirred ultrafiltration chamber equipped with a Diaflo type YM-3 membrane filter that retained proteins with molecular weights greater than 3,000 Daltons. The fast protein liquid chromatography (FPLC) system was used in combination with a Q-sepharose column to further purify the sample. The FPLC system consisted of two LKB P500 pumps (Pharmacia) combined with a model LCC-500 PLUS gradient controller (Pharmacia), a Rheodyne injection valve, a single path UV monitor (Pharmacia) and an LKB 2212 HELIRAC fraction collector. FPLC was carried out at room temperature, but the fractions collected were cooled on ice. The concentrate was applied to the Q-sepharose column (1 x 7 cm) which had previously been equilibrated with 50 mM bis-Tris propane (pH 8.5) at

room temperature. The column, was washed extensively with buffer until no further absorbance at 280 nm was detected in the eluent, whence the reductase was eluted with 10 mM NaCl. Fractions (14ml) were collected at a flow rate of 1 ml/min, desalted and concentrated by ultrafiltration as previously described. The sample was then applied to a Mimetic Orange 2 affinity chromatography column (8 x 20 mm, Affinity Chromatography Ltd.) that had previously been equilibrated with 50 mM bis-Tris propane (pH 7) at 4^0C. The column was washed with 2 column volumes of buffer. The third column volume contained the reductase activity which was collected.

Results

The specific activity of the PETN reductase was measured after the various stages of purification described above. The resulting data is set out in Table 1, from which it may be seen that in a cell free extract of *P. alcaligenes* PB2 grown on PETN as a nitrogen source, PETN reductase was present at a specific activity of 0.025 unit/mg protein while ultimately it was purified 182 fold.

Table I

Purification step	Volume (ml)	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Recovery of activity (%)	Purification factor (-fold)
Crude extract	40	361.6	9.04	0.025	100	-
Ammonium sulphate fractionation	8	31.4	3.47	0.110	39	4
Q-Sepharose ion exchange chromatography	10.5	15	0.83	0.720	9	29
Mimetic Orange 2 affinity chromatography	16	0.16	0.728	4.550	8	182

EXAMPLE 3

Characteristics of PETN reductase

pH optimum

Purified PETN reductase (50 µl, 0.5 µg protein) was incubated for 4 min at 30°C with 47 µM PETN in a range of buffers: 50 mM bis-Tris propane (pH 6.5, 7, 7.5 and 8), 50 mM 2-[N-morpholino]ethanesulphonic acid (MES) (pH 5.5, 6, 6.5) and 50 mM bis-Tris (pH 6.2, 6.3, 6.4, 6.5, 6.6, 6.7 and 6.8). The concentration of nitrite was then measured using Greiss reagent.

PETN reductase displayed a pH optimum of 6.5.

K_m of enzyme

The K_m of PETN reductase was above the maximum solubility of PETN (47 µM).

Molecular weight detemination

The molecular weight of the native enzyme was determined by the method of Andrews (Biochem. J. (1964) 91, 222-223). Measurements were carried out on a Superose 6 HR 10/30 column (1 x 30 cm). Purified PETN reductase (10 µg) was mixed with marker proteins and added to the column. The column was eluted with bis-Tris propane buffer (pH 7) and 0.4 ml fractions collected. The elution volume of PETN reductase corresponded to a molecular weight of 40,000 Daltons.

Molecular weight determination was also performed using SDS-PAGE. The purified PETN reductase ran as a distinct major band corresponding to a molecular weight of 42,000 Daltons. This similar value to that of the native enzyme implies that any effect of detergent/protein interactions in determining the molecular weight of the enzyme were minimal.

Claims

1. A PETN reductase enzyme characterised in that:
   (1) it catalyses the removal of nitrite from PETN; and
   (2) it has reductase activity specifically at the nitrate ester linkage of PETN.

2. A PETN reductase enzyme according to claim 1 further characterised by one or more of the following features:-
   (3) it has a pH optimum of 6.5; and
   (4) it has a native molecular weight of about 40,000 Daltons, as determined by gel filtration chromatography.

3. A *Pseudomonas alcaligenes* bacterial strain referred to as "PB2" and deposited as NCIMB 40718, and mutants and variants thereof capable of producing enzymic activity which degrades PETN in the presence of NADPH.

4. A process of producing an enzyme according to claim 1, which comprises culturing the *Pseudomonas alcaligenes* sp NCIMB 40718 according to claim 3, or a mutant or variant thereof, in the presence of PETN as nitrogen source, at a temperature of from 20 to 40^{0}C, disrupting the cells and recovering the enzyme from the disrupted cells.

5. A method of detecting PETN in a sample, comprising subjecting the sample to a reaction involving the removal of nitrite from PETN, the reaction being carried out in the presence of NADPH and a PETN reductase enzyme according to claim 1 or claim 2, until NADP and nitrite are produced, and detecting the occurrence of said reaction.

6. A method according to claim 5 wherein the NADP liberated in the reaction is detected.

7. A method according to claim 6 wherein NADP is detected by a bioluminescence test.

8. A method according to claim 7 wherein the bioluminescence test uses either bacterial or firefly luminescence.

9. A method according to claim 6 wherein NADP is detected amperometrically.

10. A biosensor for the detection of PETN in a sample which comprises means for contacting the sample with a PETN reductase enzyme in the presence of NADPH and means for detecting the occurrence of a reaction, catalysed by the enzyme, of PETN when PETN is present in the sample.

11. A biosensor according to claim 10 wherein the means for detecting the occurrence of a reaction comprises either a bioluminescent or an amperometric transducer.

13. A method for the bioremedial treatment of a PETN contaminated environment comprising the step of inoculating the environment with a sample of the bacterial isolate of *Pseudomonas alcaligenes* as defined in claim 3 and allowing the isolate to consume the PETN present in the environment.

14. A method according to claim 13 wherein the environment is a waste stream containing PETN.

15. A method for producing isomers of pentaerythitol di- and tri-nitrates comprising reacting PETN with the PETN reductase enzyme of claim 1 or claim 2.

The Patent Office

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Claims searched: 1-15 **Date of search:** 24 October 1995

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Databases searched:

UK Patent Office collections, including GB, EP, WO & US patent specifications, in:

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Other: ONLINE: WPI; BIOTECH/DIALOG

Documents considered to be relevant:

Category	Identity of document and relevant passage	Relevant to claims
A	JOURNAL OF GENERAL MICROBIOLOGY, Vol. 139, 1993, White and Snape, "Microbial cleavage of nitrate esters: defusing the environment", pages 1947-1957 (See pages 1953-1954)	1-15

X	Document indicating lack of novelty or inventive step	A	Document indicating technological background and/or state of the art.
Y	Document indicating lack of inventive step if combined with one or more other documents of same category.	P	Document published on or after the declared priority date but before the filing date of this invention.
&	Member of the same patent family	E	Patent document published on or after, but with priority date earlier than, the filing date of this application.